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(54) Title: A DNA SEQUENCE ENCODING THE *MAS* ONCOGENE, POLYPEPTIDES ENCODED THEREFROM, AND DIAGNOSTIC AND OTHER METHODS BASED THEREON

(57) Abstract

A DNA sequence comprising an activated oncogene, said oncogene encoding a polypeptide capable of transforming NIH3T3 cells and of inducing a tumor when injected into nude mice, said DNA sequence having a nucleotide sequence substantially as shown in Fig. 3. The invention also concerns a polypeptide molecule encoded by an activated oncogene, said molecule having the properties of transforming NIH3T3 cells and of inducing a tumor when injected into nude mice and further said polypeptide having an amino acid sequence substantially as shown in Fig. 3. Finally, this invention provides a method for treating a tumor induced by an activated *mas* oncogene.

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A DNA SEQUENCE ENCODING THE *MAS* ONCOGENE, POLYPEPTIDES ENCODED THEREFROM, AND DIAGNOSTIC AND OTHER METHODS BASED THEREON

BACKGROUND OF THE INVENTION

This work was supported by grants from the American Cancer Society, American Business for Cancer Research Foundation and the National Institutes of Health. The U.S. Government has certain rights in this invention.

Throughout this application various publications are referenced by arabic numerals within paretheses. Full citations for these references may be found at the end of the Specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art as known to those skilled therein as of the date of the invention described and claimed herein.

The first oncogenes discovered were the transforming genes of the oncogene viruses (3). The subsequent discovery that the oncogenes of retroviruses were derived from normal host cellular genes provided the first direct evidence that cellular genomes contain genes with transforming potential. More recently, the development of techniques for DNA transfer in eucaryotic cells led to the discovery by their ability to induce foci of transformed NIH3T3 cells (30). Several new oncogenes have been discovered this way including N-*ras* (43), *met* (7), *neu* (1) and possibly others (19,31,46).

To search for oncogenes which may not be capable of detection by the focus induction assay, a sensitive new bioassay for transforming genes based on the tumorigenicity in nude mice of NIH3T3 cells following cotransfection with a selectable marker and DNA from tumor cells has been employed. Using this assay, three transforming genes were derived from the DNA of the MCF-7 cell line (14). A new human oncogene called *mas* which was detected by the tumorigenicity assay following cotransfection with DNA isolated from a human epi-

dermoid carcinoma is described. This gene efficiently induces tumorigenicity and has a weak focus inducing activity in NIH3T3 cells. cDNAs containing the entire coding sequence of mas have been cloned. The mas gene encodes a protein with seven hydrophobic regions that are potential transmembrane domains, suggesting that mas is an integral membrane protein. The structure of mas protein is unique among cellular oncoproteins and may represent a new functional class.

SUMMARY OF THE INVENTION

The invention concerns a DNA sequence comprising an activated oncogene which encodes a polypeptide capable of transforming NIH3T3 cells and of inducing a tumor when injected into nude mice, said DNA sequence having a nucleotide sequence substantially as shown in Figure 3.

This invention also concerns methods for detecting tumor cells which comprise isolating genomic DNA or RNA from a cell, contacting the DNA or RNA so isolated with a detectable marker which binds specifically to at least a portion of the sequence encoding an activated oncogene of this invention, or to at least a portion of the RNA sequence encoded by an activated oncogene of this invention, and detecting the marker so bound, the presence of bound marker indicating the presence of a tumor cell.

This invention also concerns a method of determining the predisposition of a subject to a disease, which comprises isolating the genomic DNA or RNA from a cell from the subject, contacting the DNA or RNA so isolated with a detectable marker which specifically binds to at least a portion of an activated oncogene of this invention, or to a portion of the RNA encoded by an activated oncogene of this invention, and detecting the marker so bound, the presence of bound marker indicating a predisposition of the subject to the disease.

The invention also concerns a polypeptide molecule encoded by an activated oncogene, said polypeptide having the

properties of transforming NIH3T3 cells, inducing a tumor when injected into nude mice, and further said polypeptide having an amino acid sequence substantially as shown in Figure 3.

Tumor cells and tumors expressing the polypeptide of this invention may be detected with a detectable marker which specifically binds to at least a portion of the polypeptide of this invention. Further, subjects predisposed to diseases associated with the polypeptide of this invention may be identified with a detectable marker which specifically binds to at least a portion of the polypeptide.

Methods for detecting a tumor or tumor cells comprise isolating serum from a subject, contacting the serum with a detectabler marker which binds specifically to at least a portion of a polypeptide of this invention to form a marker-polypeptide complex, and detecting the marker so bound, the presence of bound marker indicating the presence of a tumor.

Finally, the invention concerns a method for treating in a subject a tumor induced by an activated mas oncogene which comprises isolating an immunoglobulin molecule which specifically binds at least a portion of a polypeptide encoded by the activated mas oncogene, attaching to the immunoglobulin molecule so isolated a substance which substantially limits the growth of a tumor or which destroys tumors to produce an antitumor immunoglobulin molecule, and contacting the tumor with an effective amount of the antitumor immunoglobulin molecule so produced, thereby limiting tumor growth or destroying the tumor.

BRIEF DESCRIPTION OF THE FIGURE

Figure 1. - Maps of restriction endonuclease sites in mas clones. Filled boxes and thick lines indicate the coding region and flanking sequences derived from the placental mas allele. Open boxes and

thin lines indicate the coding region and flanking sequences derived from the rearranged mas allele. The hatched areas indicate regions containing mouse repetitive DNA. Squiggles indicate the sites of DNA rearrangements. Restriction sites are indicated by the letters E (EcoRI), H (HpaI), S (SaII), Sm (SmaI), P (PstI), X (XholI).

Figure 2. - Relation of mas clones to genomic clone pMS424. Inserts from the indicated cDNA are aligned under the genomic sequence. The open box indicates the coding region. The arrow shows the direction of transcription. Abbreviations for restriction endonuclease sites are as described in Figure 1.

Figure 3. - Nucleotide sequence of coding and flanking regions of the mas gene. The DNA sequence was derived from cDNA clones and the genomic subclone pMS422. The amino acid sequence deduced from the coding region is shown above the DNA sequence. The inframe stop codons of the open reading frame are indicated by asterisks. The DNA sequence from the 14th nucleotide position 5' from the start ATG to the 3' end was derived from the cDNA clones. The sequence of the coding and 5' regions were determined from the genomic clone pMS422. The numbers on the right are amino acid coordinates.

Figure 4. - S1 analysis of RNA transcripts. The AccI fragment of the genomic clone

pMS422 was end-labelled and hybridized to RNA and digested with S1 nuclease. RNA used was isolated from NIH3T3 cells, MAS-133, or NIH3T3 cells transformed with the normal mas clone pHM2. Restriction endonuclease sites are indicated by the letters A (AccI), St (StuI), X (XhoI) and E (EcoRI). The StuI site is 10 bp 5' to the ATG initiation codon and includes the last base pair of the first inframe stop codon. This data indicates the colinearity between RNA transcripts and genomic sequences from the mas gene extend 5' beyond the termination codon at the StuI site.

Figure 5. - Hydrophobicity plots of the predicted mas protein. Average hydrophobic values were determined for spans of 19 residues using the method and hydrophobic values of the Kyte and Doolittle (29). The plots of the predicted mas protein, bovine rhodopsin and the alpha subunit of acetylcholine receptor are shown for comparison.

DETAILED DESCRIPTION OF THE INVENTION

A DNA sequence comprising an activated oncogene has been isolated which encodes a polypeptide capable of transforming NIH3T3 cells and of inducing a tumor when injected into nude mice. The DNA sequence has a nucleotide sequence substantially as shown in Figure 3.

The DNA sequence of this invention encodes a mas oncogene. The sequence may be isolated from a variety of sources, although the presently preferred sequence encodes the human mas gene. The polypeptide produced by the trans-

cription of the gene and the translation of the gene product will vary with the initial DNA sequence.

A method of detecting a tumor cell which contains the DNA sequence of this invention is described. The method comprises isolating genomic DNA from a cell, contacting the DNA isolated from the cell with a detectable marker which binds specifically to at least a portion of the DNA sequence of this invention which encodes an activated oncogene and detecting the marker so bound. The presence of bound marker indicates the presence of a tumor cell.

The detectable marker may be a labelled DNA sequence, including a labelled cDNA sequence, having a nucleotide sequence complementary to at least a portion of the DNA sequence of this invention, produced by methods known to those skilled in the subject art.

The detectable marker may also be labelled ribonucleotide (RNA) sequence having a nucleotide sequence complementary to at least a portion of the DNA sequence of this invention, and may be isolated by methods known to those skilled in the art.

Detectable markers of this invention will be labelled with commonly employed radioactive labels, i.e ^{32}P, although other labels may be employed. The markers will be detected by autoradiographic, spectrophotometric or other means known in the art.

A method of detecting a tumor cell which contains RNA encoded by a DNA sequence of this invention is described. The method comprises isolating RNA from a cell, contacting the RNA isolated from the cell with a detectable marker which binds specifically to at least a portion of the RNA encoded by an activated oncogene and detecting the marker so bound. The presence of bound marker indicates the presence of a tumor cell.

The detectable marker may be a labelled DNA sequence, including a labelled cDNA sequence, having a nucleo-

tide sequence complementary to at least a portion of the DNA sequence of this invention, produced by methods known to those skilled in the subject art.

The detectable marker may also be labelled ribonucleotide (RNA) sequence having a nucleotide sequence complementary to at least a portion of the DNA sequence of this invention, and may be isolated by methods known to those skilled in the art.

Detectable markers of this invention will be labelled with commonly employed radioactive labels, i.e. ^{32}P, although other labels may be employed. The markers will be detected by autoradiographic, spectrophotometric or other means known in the art.

Methods to be used for the isolation of DNA or RNA for the practice of this invention are well known in the subject art (cf. ref. 13).

A method of determining the predisposition of a subject to a disease associated with DNA sequence of this invention is presented. The method involves isolating the genomic DNA of a cell from the subject, contacting the DNA so isolated with a detectable marker which specifically binds to at least a portion of an activated oncogene of this invention and detecting the marker so bound. The presence of bound marker indicates a predisposition of the subject to the disease.

The detectable marker may be a labelled DNA sequence, including a labelled cDNA sequence, having a nucleotide sequence complementary to at least a portion of the DNA sequence of this invention, produced by methods known to those skilled in the subject art.

The detectable marker may also be a labelled ribonucleotide (RNA) sequence having a nucleotide sequence complementary to at least a portion of the DNA sequence of this invention, and may be isolated by methods known to those skilled in the art.

Detectable markers of this invention will be labelled with commonly employed radioactive labels, i.e. ^{32}P, although other labels may be employed. The markers will be detected by autoradiographic, spectrophotometric or other means known in the art.

A method of determining the predisposition of a subject to a disease is disclosed which comprises isolating the RNA of a cell from the subject, contacting the RNA so isolated with a detectable marker which specifically binds to at least a portion of the RNA encoded by an activated oncogene of this invention and detecting the marker so bound, the presence of bound marker indicating a predisposition of the subject to the disease.

The detectable marker may be a labelled DNA sequence, including a labelled cDNA sequence, having a nucleotide sequence complementary to at least a portion of the DNA sequence of this invention, produced by methods known to those skilled in the subject art.

The detectable marker may also be labelled ribonucleotide (RNA) sequence having a nucleotide sequence complementary to at least a portion of the DNA sequence of this invention, and may be isolated by methods known to those skilled in the art.

Detectable markers of this invention will be labelled with commonly employed radioactive labels, i.e. ^{32}P, although other labels may be employed. The markers will be detected by autoradiographic, spectrophotometric or other means known in the art.

A polypeptide molecule encoded by an activated oncongene is also provided by this invention. The polypeptide has the properties of transforming NIH3T3 cells and of inducing a tumor when injected into nude mice. The polypeptide has an amino acid sequence substantially as shown in Figure 3.

The presently preferred polypeptide will be encoded

by a mas oncogene, and will be expressed in a human, although the polypeptide may be expressed in a variety of other organisms.

The polypeptide of this invention may be obtained by synthetic means, i.e. chemical synthesis of the polypeptide from its component amino acids, by methods known to those skilled in the art. In the presently preferred embodiment, the polypeptide may be obtained by isolating it from cells expressing the mas gene, i.e. a cloned mas gene in a bacterial cell, or by in vitro translation of the mRNA encoded by the mas gene to produce the polypeptide of this invention. Techniques for the isolation of polypeptides by these means are well known to those skilled in the art.

A method of detecting a tumor cell is also provided. The method involves isolating a cell, contacting the cell with a detectable marker which binds specifically to at least a portion of a polypeptide of this invention and detecting the presence of marker bound to the cell. The presence of marker so bound indicates that the cell may be a tumor cell.

The detectable marker will preferably be a labelled immunoglobulin molecule, although other markers known in the art may be employed. The immunoglobulin molecule may be an antibody produced by contacting the immune system of an animal with at least a portion of the polypeptide of this invention, or with a synthetic amino acid sequence substantially similar to a portion of a polypeptide of this invention. The antibody molecule may also be produced by a combination of recombinant DNA techniques with other techniques known in the art.

Detectable markers of this invention will be commonly employed markers such as heavy metals, radioactive, e.g. ^{35}S, fluorescent, e.g. fluorescein, or enzymatic, e.g. peroxidase. Detection of the labelled markers may be carried out by autoradiographic, spectrophotometric, or colorimetric techniques or by other methods known in the art.

A method for detecting a tumor in a subject is also presented. This method comprises contacting the tumor with a detectable marker which specifically binds at least a portion of a polypeptide of this invention and detecting the marker so bound. The presence of bound marker indicates the presence of a tumor.

A presently preferred method comprises introducing into the bloodstream of the patient a detectable amount of the marker such that the marker contacts and binds to a tumor expressing the polypeptide encoded by an activated oncogene, the tumor being detectable thereby.

The presently preferred marker is a labelled immunoglobulin molecule, although other markers known in the art may be employed. The immunoglobulin molecule may be an antibody produced by contacting the immune system of an animal with at least a portion of the polypeptide of this invention, or with a synthetic amino acid sequence substantially similar to a portion of a polypeptide of this invention. The antibody may also be produced by a combination of recombinant DNA techniques with other techniques known in the art.

Presently preferred labels for the antibody molecules are radioopaque labels, i.e. heavy metals, or enzymatic markers, known in the art.

A serum-based method for detecting a tumor in a subject is also presented. The method comprises isolating serum from the subject, contacting the serum with a first detectable marker which binds specifically to at least a portion of a polypeptide of this invention and detecting the marker so bound. The presence of bound marker indicates the presence of a tumor.

The first detectable marker is preferably a labelled antibody which may be free, i.e. in a solution, or may be bound to a matrix, i.e. a matrix such as polystyrene beads or the wall of a tube.

Presently preferred labels for the antibody mole-

cule are radiolabels, i.e. ^{35}S, heavy metals, or enzymatic labels. The bound antibodies will be detectable by autoradiography, scintillation counting or by colorimetry or by various other means.

The second detectable marker of this invention will also be a labelled antibody molecule and may specifically bind to the polypeptide or to the first marker or to a combination. The second marker will be labelled by radiolabels, heavy metals or enzymes, and detectable by means similar to those used to detect the first marker. The second marker may also be detectable by visual inspection if it causes precipitation of the first marker-polypeptide complex.

Finally, a method for treating in a subject a tumor induced by an activated mas oncogene is disclosed. The method comprises isolating an immunoglobulin molecule which specifically binds to at least a portion of a polypeptide encoded by the activated mas oncogene, attaching to the immunoglobulin molecule so isolated a substance which substantially limits the growth of a tumor or which destroys tumors to produce an antitumor immunoglobulin molecule, and contacting the tumor with an effective amount of the antitumor immunoglobulin molecule so produced, thereby limiting tumor growth or destroying the tumor.

The immunoglobulin molecule is preferably obtained by contacting the immune system of an animal with at least a portion of a polypeptide encoded by the associated mas oncogene, or with a synthetic amino acid sequence substantially similar to a portion of the polypeptide, to produce a specific antibody molecule, and isolating the antibody therefrom. The immunoglobulin molecule may also be produced by a combination of recombinant DNA techniques and other techniques known in the art.

Substances which substantially limit the growth of a tumor or which destroy tumors are known in the art, and may be molecules such as interferon, tumor necrosis factors,

or radioactively labelled amino acids.

MATERIALS AND METHODS

FOCUS AND TUMORIGENICITY ASSAYS

High molecular weight DNA was purified from cell lines (37) and solid tumors (14) as described. DNA transfer into NIH3T3 cells were performed by a modified calcium phosphate precipitation method (50). Focus assays (37) and tumorigenicity experiments (14) were performed as previously described. The plasmids pT24 (containing the activated human H-*ras*^{val12} gene) and pKOneo (containing a neomycin/G418 antibiotic resistance gene) were previously described (13,14).

CONSTRUCTION OF LIBRARIES

Genomic libraries were constructed in the cosmid vector pHC79 (23,33) from EcoRI partially cleaved DNA and were screened by colony filter hybridization (22). A cDNA library was constructed in lambda gt10 (25) from purified poly A + mRNA (33) from the MAS-133 cell line. The cDNA library was screened by plaque hybridization (52).

DNA ANALYSIS

Southern blots were performed as previously described (43). S1 mapping was done by a modification of the Berk-Sharp method (48). DNA sequences were determined in both orientations by the dideoxy method of Sanger et al. (40) as modified by Biggins et al. (2). DNA and protein homology searches were performed using a previously developed algorithm (18), and the Protein Indentification Resource (National Biomedical Research Foundation, Georgetown University Medical Center, Washington, D.C.) and GENBANK (Bolt, Beranek and Newman, Inc., Cambridge, Mass.) data banks.

RESULTS

ISOLATION OF THE MAS ONCOGENE

The *mas* oncogene was detected using the cotransfection and tumorigenicity assay as previously described (14). The *mas* gene was then isolated from cotransformed cells using established methods. DNA from a human epidermoid car-

cinoma was used to cotransfect NIH3T3 cells with the plasmid pKOneo and transfected cells were selected with neomycin analog G418. In one experiment, one of the six nude mice injected with these cells developed a "primary" tumor within four weeks. Nude mice injected with cells cotransfected with DNA isolated from this primary tumor developed "secondary" tumors within two weeks. After a third round, DNA was purified from a "tertiary" tumor and a genomic library was constructed in the cosmid vector pHC79. Four overlapping cosmid clones which contained human DNA were isolated by filter hybridization to total human genomic DNA (20,42). Characterization of these cosmids by restriction mapping and Southern hybridization to total human DNA revealed that one of the cosmids, pMAS1, contains a 22 kbp stretch of human DNA flanked by mouse DNA on both sides (Figure 1).

The pMAS1 cosmid was tested by the cotransfection and tumorigenicity assay to determine if it contained transforming potential. Nude mice developed tumors within two weeks after injection of cells which had been cotransformed with pMAS1 and pKOneo and selected for resistance to G418 (see Table 1). Furthermore, in our standard focus assay, NIH3T3 cells transfected with pMAS1 formed foci within 16 days. The foci of cells transformed with pMAS1 are unlike foci of cells transformed with the activated human H-ras^{val12} oncogene, isolated from the T24 bladder carcinoma cell line. They appear at a lower frequency about 4-6 days later (Table 2). The foci induced by pMAS1 are characterized not by an abnormal morphology of the constituent cells, but by an exceedingly high cell density.

To define the regions of pMAS1 essential for transforming activity, various restrictions endonuclease digests of pMAS1 were tested by the NIH3T3 focus assay. When

TABLE 1. TUMORIGENICITY ASSAYS

	Mean tumor diameter (mm) after week:					
Test Plasmid	1	2	3	4	5	6
pMAS-1	0.0	11.1	19.6			
	0.0	9.8	16.0			
	0.0	10.9	17.0			
	0.0	10.6	18.0			
pHM-2	0.0	0.0	4.5	10.6	11.2	
	0.0	0.0	5.4	10.6	14.0	
	0.0	0.0	0.0	5.8	13.6	
	0.0	0.0	0.0	8.7	13.7	
	0.0	0.0	0.0	0.0	5.0	9.8
	0.0	0.0	0.0	0.0	0.0	0.0
None	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0
pT24	10.6	22.8				
	9.0	22.8				

Tumorigenicity assays were performed as previously described (14) (1984). NIH3T3 cells ($8x10^5$ cells/plate) were cotransformed with 200 ng pKOneo, 30 ng of high molecular weight NIH3T3 DNA and test plasmid (300 ng of pMAS1, 300 ng of pHM2, or 50 ng of pT24) per plate. Following transfection, cells were split 1:5 and selected for resistance to antibiotic G418. After 2 weeks the cells were confluent and were injected into nude mice (10^7 cells per mouse). Tumor formation at the site of injection was followed for six weeks and the mean tumor diameter was measured. Each line in the above table represents a single animal injected with independently cotransformed pools of cells.

pMAS1 was cut with EcoRI or SalI the transforming potential was destroyed, indicating that regions including one or more of each of these sites are essential for transforming activity (Table 2). In contrast, pMAS1 DNA cut with SmaI or XhoI retained its transforming ability. A 7.3 kbp region of pMAS1, which is defined by SmaI and XhoI sites and contains single EcoRI and SalI sites, is capable of transforming NIH3T3 cells. This region was subcloned into pUC8 to generate pMS422 (Figure 1).

ACTIVATION OF THE MAS ONCOGENE BY REARRANGEMENT

Comparison of Southern blots of normal human DNA with DNA derived from the tertiary nude mouse tumor revealed a difference in the size and intensity of EcoRI restriction fragments homologous to mas suggesting that the mas gene was rearranged and amplified in transformants. To determine the nature of the DNA rearrangement, clones containing homology to a region of pMAS1 were isolated from a human placenta cosmid library. The probe used was the 2.2 kbp SalI-EcoRI restriction fragment which does not contain Alu-repetitive sequences and includes the 3' portion of the mas coding sequence (see next section). Three independent overlapping clones desginated pHM1, pHM2 and pHM3 were obtained. Comparison of maps of restriction endonuclease sites in pHM2 and pMAS1 confirm that there is a break in homology localized between the EcoRI and HpaI sites in the 5' non-coding region of the mas gene (Figure 1). Although this break is not in the mas coding region (see next section), it does occur in a region essential for transformation of NIH3T3 cells by the mas gene (see above and Table 2). This suggests that the rearrangement found in the transformant is of functional significance.

TABLE II. FOCUS ASSAY

Exp't A	DNA	ng/plate	foci/ng (2 wks.)
	pMAS1	200	0.105
	pHM2	200	<0.0025
	pHM2	1000	<0.0005
	pT24	50	2.0
Exp't B	DNA	ng/plate	foci/ng (3 wks.)
	pMAS1	100	0.36
	pMAS1/SmaI	100	0.41
	pMAS1/XhoI	100	0.50
	pMAS1/EcoRI	100	<0.01
	pMAS1/SalI	100	<0.01
Exp't C	DNA	ng/plate	foci/ng (3 wks.)
	pMS422	400	0.61
	pMS424	400	0.50
	pGW34	400	0.60
	pHMS4	400	<0.001

Focus assays in NIH3T3 cells were performed as previously described (Perucho et al, 1981). NIH3T3 cells were transformed with the indicated amount of test DNAs, either cleaved with the indicated restriction enzymes (Exp't B), or uncleaved (Exp't A and Exp't C). High molecular weight NIH3T3 DNA (30 ng/plate) was used as carrier DNA. After the indicated times, the number of foci were scored and the number of foci/ng of test DNA was calculated. See Figure 1 for a description of the plasmid clones.

The cosmid pHM2, which contains the normal human homolog of mas, was tested to determine if it has transforming activity. By the standard transfection assay described above, pHM2 did not induce foci of transformed NIH3T3 cells even after four weeks. However, pHM2 did induce tumors in a cotransfection and tumorigenicity assay, although with a longer lag time than pMAS1 (see Table 1). Therefore, the normal mas clones, pHM2, has a weak biological transforming activity detected by our tumorigenicity assays.

To test if the DNA rearrangement was responsible for activation of mas transforming potential, the hybrid clone pGW34 was constructed, which contains the 5' non-coding region of the rearranged gene and the entire coding region from the normal human homolog (see Figure 1 and next section). This hybrid clone had focus inducing activity similar to the mas clone, pMS422 (Table 2). In contrast, pHMS4, a subclone of the normal placenta clone (Figure 1), did not induce foci, suggesting that the DNA rearrangement is responsible for activation of mas transforming potential. Since this rearrangement lies outside of coding sequence, activation of the mas gene may result from overproduction or inappropriate production of its normal product. However, in order to make a definitive conclusion the nature of the normal gene transcript must be determined.

Finally, experiments were undertaken to determine if the mas gene was activated in the donor tumor DNA or, rather, became activated during cotransfection into NIH3T3 cells. Southern blotting experiments demonstrated that the mas genes resident in the original human epidermoid carcinoma did not have the rearranged structure found in the NIH3T3 cotransformant, nor were they amplified. Moreover, the transfer of the mas gene from the original tumor DNA has not been observed in other cotransformants. Thus, there is no evidence that the mas gene was active in the original human tumor. Rather, it is likely that the mas gene became rear-

ranged and activated during gene transfer from the original human tumor DNA.

ORGANIZATION AND SEQUENCE OF THE MAS GENE TRANSCRIPT

In order to define the transcription unit and coding potential of the mas gene, cDNAs complementary to mas mRNA were cloned. Poly A+ mRNA was purified from a cell line, MAS-133, derived from the nude mouse tumor from which pMAS1 was isolated. Blot hybridization of this RNA with the mas gene insert from pMS422 revealed a homologous mRNA approximately 2.5 kbp in length (data not shown). A cDNA library was constructed from this poly A+ RNA in the lambda gt10 vector and was screened for homology to pMS422. Sixteen overlapping cDNA clones were isolated and characterized by restriction endonuclease site mapping and DNA sequencing (see Figure 2). From sequence data a composite nucleotide sequence was assembled which contains a complete open reading frame of 975 bp (Figure 3). The first ATG in this reading frame is preceeded by an inframe stop codon at position -12. The entire open reading frame was obtained on a single cDNA clones, pMC130. The orientation of transcription, as shown in Figure 2 was postulated from this data.

Comparison of the restriction endonuclease cleavage sites of genomic and cDNA sequences indicated that the cDNA is entirely colinear with genomic sequences. This observation was subsequently confirmed for the coding region by direct nucleotide sequencing of the homologous region of the genomic DNA clone pMS422 (see Figure 3). By the S1 analysis it was determined that the colinearity of the mRNA with the genomic DNA extends in the 5' direction approximately 45 bases beyond the first ATG in the coding region (Figure 4). The site which marks the beginning of colinearity between RNA and genomic sequences may reflect a transcription initiation site or a splice site. Since no cDNA clones containing poly A were found, it is likely that mas transcripts extend beyond the 3' end of the cDNA clone pMC142 (see Figure 2).

In order to learn more about the organization of the normal mas gene and the events which can lead to its activation, RNA was examined in cells which were cotransformed with the cosmid pHM2, selected from tumorigenicity in nude mice, and subsequently placed back into culture. Total RNA was prepared from these cells and analyzed in the manner described for MAS-133 cells (see Figure 4). This analysis indicates that transcripts of mas in these cells are also colinear with genomic DNA 5' to the open reading frame already identified. Hence, the same protein is probably encoded in these cells as in MAS-133.

PREDICTED PRIMARY AND SECONDARY STRUCTURE OF THE MAS PROTEIN

The complete nucleotide sequence of the coding region determined from mas cDNAs and the corresponding predicted amino acid sequence of 325 amino acids are shown in Figure 3. The deduced amino acid sequence does not share significant homology with any published sequence. Analysis of the hydrophobicity plot of the sequence by the method of Kyte and Doolittle (29) reveals that the mas protein has seven distinct hydrophobic regions (Figure 5). Computational methods based on hydrophobicity profiles have been developed to distinguish transmembrane regions from hydrophobic internal regions of globular proteins (10). A recently developed algorithm correctly predicts membrane spanning segments in many proteins, including the seven segments in bacteriorhodopsin, and correctly disqualifies hydrophobic regions from many soluble proteins (11). This algorithm predicts seven transmembrane domains in the mas gene corresponding to the seven hydrophobic regions. Each of the hydrophobic regions is separated by hydrophilic regions which contain a predicted betaturn secondary structure (6). Both the amino acid and carboxy terminal ends of the mas protein are hydrophilic. This analysis strongly suggests that the mas protein is an integral membrane protein with many transmembrane domains.

Several proteins which span the membrane multiple times have been identified and studied. These include bacteriorhodopsin and eucaryotic visual rhodopsins (36), lactose permease (16), the acetylcholine receptor (38), the sodium ion channel (35), the $(Na^+ + K^+)$ATPase (27,44), the Ca^{2+}-ATPase (32), and the erythrocyte anion exchange protein (26). In Figure 5 the hydrophobicity plots of bovine rhodopsin and the alpha-subunit of acetlycholine receptor is shown for comparison with mas protein. There is a striking similarity in the hydrophobicity patterns of mas and rhodopsin, which may reflect structural and functional similarities in these proteins.

The mas protein does not contain an N-terminal hydrophobic signal sequence characteristic of many membrane proteins (49). However, some proteins with multiple transmembrane domains such as bovine rhodopsin, the erythrocyte anion exchange protein, and the sodium channel protein, lack amino-terminal signal sequences. In the first two cases, insertion into the membrane is cotranslational and requires internal signal sequences (5,17). Since the mas protein does not contain an amino terminal hydrophobic signal sequence, its integration into the membrane may also depend on internal signal sequences or, alternatively, it may spontaneously insert into the membrane due to its hydrophobic nature (4,12).

It has been established that the tripeptide sequence Asn-X-Thr/Ser is a site for N-glycosylation in secreted and membrane proteins (28), although not all of these sequences are glycosylated (45). There are four potential sites for N-glycosylation in the predicted protein sequence of mas at positions 5, 16, 22 and 272. The first three of these sites are clustered in the first hydrophilic amino terminal region, while the fourth site is in the seventh hydrophobic domain.

DISCUSSION

The cotransfection and tumorigenicity assay was developed to search for transforming genes from tumor cells which may not be readily detected by the standard focus assay. Using this assay, three transforming genes were previously isolated from MCF-7 cell line (14). One of these gene is a normal N-ras gene, which is amplified in MCF-7 DNA. Another, designated mcf2, is currently being investigated, and the third gene, mcf3, is the human homolog of v-ros (Birchmeier et al., manuscript in preparation). In the later case, the human ros gene was rearranged during or after gene transfer, probably resulting in its activation. A similar event appears to have occurred in the activation of the mas gene. The mas gene was found to be rearranged and amplified in the tertiary nude mouse tumor DNA. In contrast, the mas genes of the original human tumor DNA, used in the first round of cotransfection experiments, have the normal configuration and are not amplified. Although the normal human mas gene has weak tumor inducing activity, it has no detectable focus inducing activity. By contrast, the rearranged mas gene has strong tumor inducing activity and can induce foci of NIH3T3 cells. Analysis by chimeric gene construction indicates that a similar rearrangement can activate the normal placental allele. Therefore, it was concluded that the cotransfection and tumorigenicity assay has a propensity for detecting those proto-oncogenes which can induce tumorigenicity in NIH3T3 cells by arrangement and/or amplification following cotransfection. In this respect, the NIH3T3 cotransfection and tumorigenicity assay differs considerably from the NIH3T3 focus assay, which very rarely scores positive with DNA which does not already contain an activated oncogene.

It is not yet precisely understood how the mas gene became activated during gene transfer. In transformants, the mas gene has been rearranged, but this rearrange-

ment does not appear to involve the coding domain. It is clear that the oncogenic potential of a normal placental allele can be activated by reconstructing a chimeric gene which replaces the normal 5' sequence with a sequence 5' to the coding region of the rearranged gene. This chimeric gene leaves intact the long open reading frame of the placental allele. Thus it seems likely that transformation by mas results from inappropriate expression of a normal gene product. However, the possibility that the rearranged alleles have an altered splicing pattern which results in an altered protein product cannot be excluded. To completely resolve this problem mas transcripts in a normal cell must be identified. However, transcripts of the mas gene in cells co-transformed with the normal allele have been examined. S1 analysis indicates that in such cells we can exclude the existence of an additional N-terminal coding domain.

There are currently about 30 known viral or cellular oncogenes which code for proteins associated with the plasma membrane (30). Three oncogenes, v-erb-B, v-fms and v-ros code for proteins with single transmembrane domains and are probably related to growth factor receptors (9,21,34). While ras and src do not encode transmembrane domains, they are associated with the membrane and have fatty acid residues which are added to the proteins by a posttranslational modification (41,8,51). Most of these membrane associated oncoproteins, with the exception of the ras proteins, have tyrosine kinase activities which have been implicated in transformation (24). Although there is no direct proof that tyrosine phosphorylation is responsible for cellular transformation, there is a good correlation between the transforming abilities and tyrosine phosphorylation activities of viral transforming proteins.

In contrast to the structure of the membrane associated oncogene products discussed above, the structure of the mas gene product implied from the cDNA sequence is very

different. A computer search through the Protein Identification Resource and GENBANK data bases (see Experimental Procedures) found no significant homology between mas and any known DNA or protein sequences, including protein kinases or ras proteins. The hydrophobicity plot of the amino acid sequence indicates that there are seven very hydrophobic regions which are potential transmembrane domains. This strongly suggests that the mas protein is an integral membrane protein which may cross the plasma membrane many times. Only one transforming gene has previously been described which encodes a protein with multiple transmembrane domains. This gene, encoded by the Epstein-Barr virus, codes for a plasma membrane protein (LMP) with six potential transmembrane domains (15) and has recently been shown to be capable of transforming Rat-1 cells (47). Unlike the mas protein, LMP has a long carboxy terminal hydrophilic region. Moreover, unlike the mas gene, the LMP gene does not render NIH3T3 cells tumorigenic.

While the structure of mas is unlike the canonical structure of the most of the known hormone receptors it is similar to a class of proteins which include the acetylocholine receptor and the visual rhodopsins. The acetylcholine receptor functions as a hormonally regulated ion channel. Visual rhodopsin is a light receptor which functions to activate transducin, an intracellular guanine nucleotide binding protein. The mas gene may encode a receptor which activates a critical component in a growth regulatory pathway, perhaps by serving in signal transduction or as a membrane channel. The unique nature of mas leads to the suspicion that it may provide a new link in understanding growth control.

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THE EMBODIMENTS OF THE INVENTION IN WHICH AN EXCLUSIVE PROPERTY OR PRIVILEGE IS CLAIMED ARE DEFINED AS FOLLOWS:-

1. A DNA sequence comprising an activated oncogene, said oncogene encoding a polypeptide capable of transforming NIH3T3 cells and of inducing a tumor when injected into nude mice, said DNA sequence having a nucleotide sequence substantially as shown in Figure 3.

2. A DNA sequence of Claim 1, wherein the activated oncogene is the mas oncogene.

3. An activated oncogene of Claim 2, wherein the mas oncogene is from a human.

4. A method of detecting a tumor cell which comprises isolating genomic DNA from a cell, contacting the DNA so isolated with a detectable marker which binds specifically to at least a portion of the sequence encoding an activated oncogene of Claim 1, and detecting the marker so bound, the presence of bound marker indicating the presence of a tumor cell.

5. A method of Claim 4, wherein the detectable marker is a nucleotide sequence complementary to at least a portion of the activated oncogene.

6. A method of Claim 5, wherein the nucleotide sequence is a complementary deoxyribonucleotide (cDNA) sequence.

7. A method of Claim 5, wherein the nucleotide sequence is a ribonucleotide (RNA) sequence.

8. A method of Claim 5, wherein the detectable marker is labelled with a radiolabeled nucleotide.

9. A method of Claim 4, wherein the detecting is by autoradiography.

10. A method of Claim 4, wherein the detecting is by spectrophotometry.

11. A method of Claim 4, wherein the detecting is by colorimetry.

12. A method of detecting a tumor cell which comprises isolating RNA from a cell, contacting the RNA so isolated with a detectable marker which binds specifically to at least a portion of the RNA sequence encoded by an activated oncogene of Claim 1, and detecting the marker so bound, the presence of bound marker indicating the presence of a tumor cell.

13. A method of Claim 12, wherein the detectable marker is a nucleotide sequence complementary to at least a portion of the activated oncogene.

14. A method of Claim 12, wherein the nucleotide sequence is a complementary deoxyribonucleotide (cDNA) sequence.

15. A method of Claim 12, wherein the nucleotide sequence is a ribonucleotide (RNA) sequence.

16. A method of Claim 12, wherein the detectable marker is labelled with a radiolabeled nucleotide.

17. A method of Claim 12, wherein the detecting is by autoradiography.

18. A method of Claim 12, wherein the detecting is by spectrophotometry.

19. A method of Claim 12, wherein the detecting is by colorimetry.

20. A method of determining the predisposition of a subject to a disease, which comprises isolating the genomic DNA of a cell from the subject, contacting the DNA so isolated with a detectable marker which specifically binds to at least a portion of an activated oncogene of Claim 1 and detecting the marker so bound, the presence of bound marker indicating a predisposition of the subject to the disease.

21. A method of Claim 20, wherein the detectable marker is a nucleotide sequence complementary to at least a portion of the activated oncogene.

22. A method of Claim 20, wherein the nucleotide sequence is a complementary deoxyribonucleotide (cDNA) sequence.

23. A method of Claim 20, wherein the nucleotide sequence is a ribonucleotide (RNA) sequence.

24. A method of Claim 20, wherein the detectable marker is labelled with a radiolabeled nucleotide.

25. A method of Claim 20, wherein the detecting is by autoradiography.

26. A method of Claim 20, wherein the detecting is by spectrophotometry.

27. A method of Claim 20, wherein the detecting is by colorimetry.

28. A method of determining the predisposition of a subject to a disease, which comprises isolating the RNA from a cell from the subject, contacting the RNA so isolated with a detectable marker which specifically binds to at least a portion of the RNA encoded by an activated oncogene of Claim 1 and detecting the marker so bound, the presence of bound marker indicating a predisposition of the subject to the disease.

29. A method of Claim 28, wherein the detectable marker is a nucleotide sequence complementary to at least a portion of the activated oncogene.

30. A method of Claim 28, wherein the nucleotide sequence is a complementary deoxyribonucleotide (cDNA) sequence.

31. A method of Claim 28, wherein the nucleotide sequence is a ribonucleotide (RNA) sequence.

32. A method of Claim 28, wherein the detectable marker is labelled with a radiolabeled nucleotide.

33. A method of Claim 28, wherein the detecting is by autoradiography.

34. A method of Claim 28, wherein the detecting is by spectrophotometry.

35. A method of Claim 28, wherein the detecting is by colorimetry.

36. A polypeptide molecule encoded by an activated oncogene, said molecule having the properties of transforming NIH3T3 cells, inducing a tumor when injected into nude mice, and further said polypeptide having an amino acid sequence substantially as shown in Figure 3.

37. A polypeptide of Claim 36, wherein the activated oncogene is the mas oncogene.

38. A polypeptide of Claim 36, wherein the mas oncogene is from a human.

39. A method of detecting a tumor cell which comprises isolating a cell, contacting the cell with a detectable marker which binds specifically to at least a portion of a polypeptide of Claim 36 and detecting the presence of marker so bound to the cell, the presence of marker so bound indicating a tumor cell.

40. A method of Claim 39, wherein the detectable marker is a labelled immunoglobulin molecule.

41. A method of Claim 40, wherein the labelled immunoglobulin is labelled with a radionucleotide.

42. A method of Claim 40, wherein the labelled immunoglobulin is labelled with an enzyme.

43. A method of Claim 39, wherein the detecting is by autoradiography.

44. A method of Claim 39, wherein the detecting is by spectrophotometry.

45. A method of Claim 39, wherein the detecting is by colorimetry.

46. A method for detecting a tumor in a subject which comprises contacting the tumor with a detectable marker which specifically binds at least a portion of a polypeptide of Claim 36, and detecting the marker so bound, the presence of bound marker indicating the presence of a tumor.

47. A method of Claim 46, wherein the subject is a human.

48. A method of Claim 46, wherein the detectable marker is a labelled immunoglobulin molecule.

49. A method of Claim 48, wherein the immunoglobulin is labelled with a radioactive substance.

50. A method of Claim 48, wherein the immunoglobulin is labelled with a heavy metal substance.

51. A method of Claim 48, wherein the immunoglobulin is labelled with an enzyme.

52. A method of Claim 46, wherein the contacting comprises introducing into the bloodstream of the patient a detectable amount of the marker such that the marker contacts and binds to a tumor expressing the polypeptide encoded by an activated oncogene, the tumor being detectable thereby.

53. A method for detecting a tumor in a subject which comprises isolating serum from the subject, contacting the serum with a first detectable marker which binds specifically to at least a portion of a polypeptide of Claim 36 to form a first marker-polypeptide complex and detecting the marker so bound, the presence of bound marker indicating the presence of a tumor.

54. A method of Claim 53, wherein the detectable marker is a labelled immunoglobulin molecule.

55. A method of Claim 54, wherein the immunoglobulin is labelled with a radioactive substance..

56. A method of Claim 53, wherein a second detectable marker which specifically binds to at least a portion of the polypeptide is contacted with the first marker-polypeptide complex.

57. A method of Claim 53, wherein a second detectable marker which specifically binds to at least a portion of the first marker is contacted with the first marker-polypeptide complex.

58. A method of determining the predisposition of a subject to a disease, which comprises isolating serum from the subject, contacting the serum with a first detectable marker which binds specifically to at least a portion of a polypeptide of Claim 36 and detecting the marker so bound, the presence of bound marker indicating a predisposition of the subject to the disease.

59. A method of Claim 58, wherein the subject is a human.

60. A method of Claim 58, wherein the detectable marker is a labelled immunoglobulin molecule.

61. A method of Claim 58, wherein the second detectable marker which specifically binds to at least a portion of the polypeptide is contacted with the polypeptide.

62. A method of Claim 58, wherein the second detectable marker which specifically binds to at least a portion of the first marker is contacted with the polypeptide.

63. A tumor cell surfae antigen comprising at least a portion of the polypeptide of Claim 36.

64. A method for treating in a subject a tumor induced by an activated mas oncogene which comprises isolating an immunoglobulin molecule which specifically binds at least a portion of a polypeptide encoded by the activated mas oncogene, attaching to the immunoglobulin molecule so isolated a substance which substantially limits the growth of a tumor or which destroys tumors to produce an antitumor immunoglobulin molecule, and contacting the tumor with an effective amount of the antitumor immunoglobulin so produced, thereby limiting tumor growth or destroying the tumor.

Fig. 1
1/5

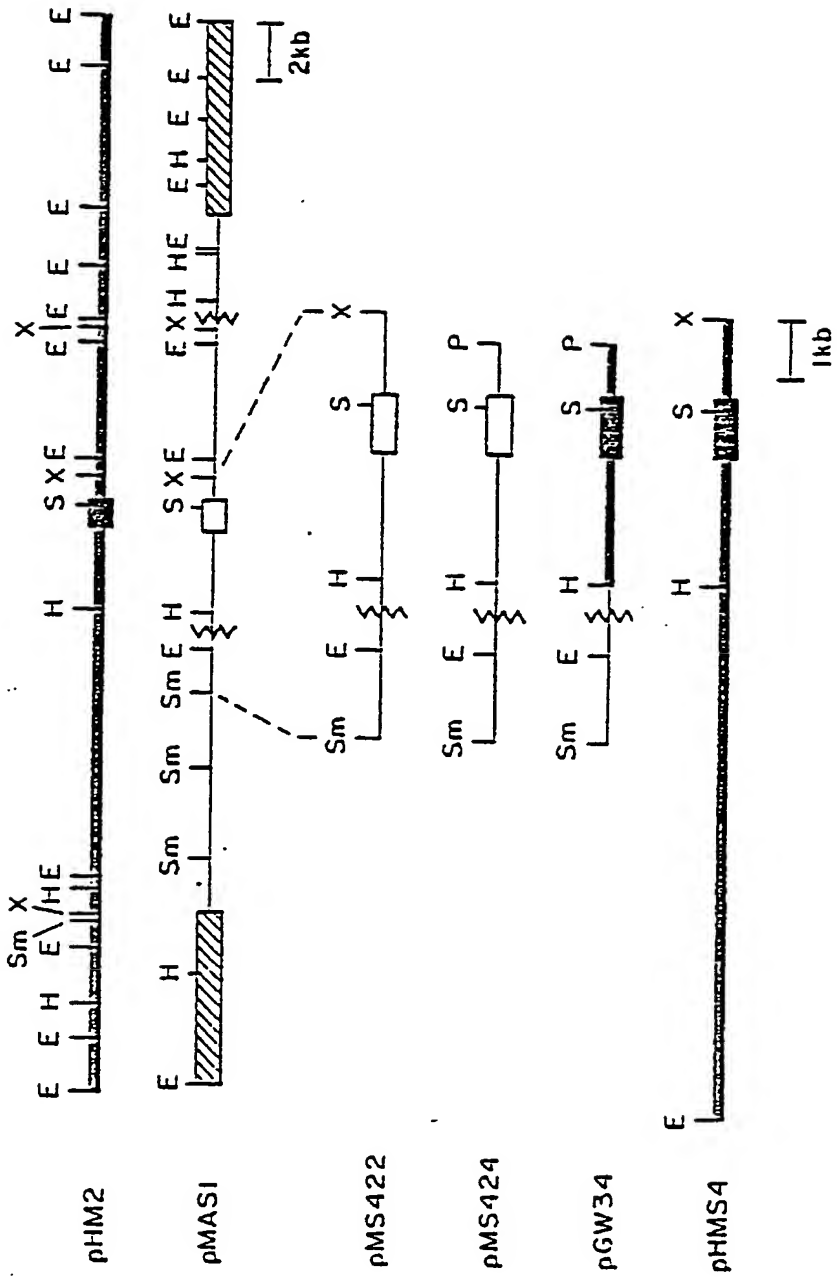

Fig. 2

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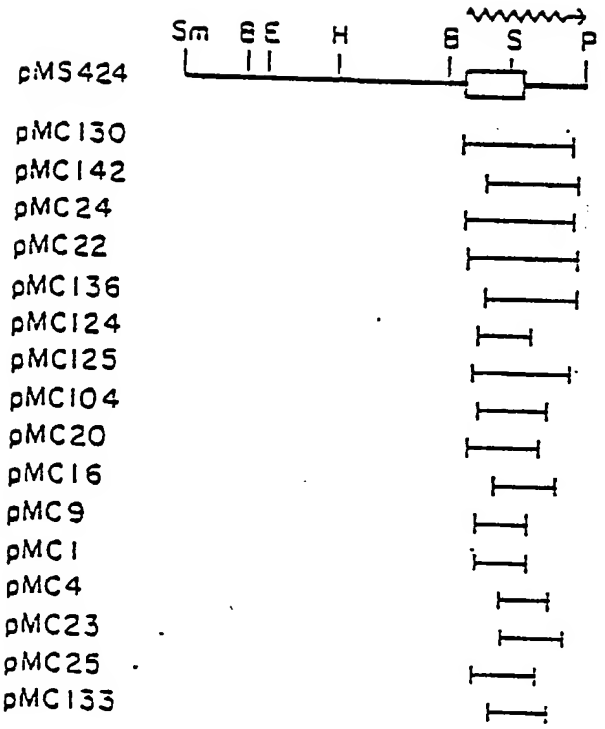
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pMC23
pMC25
pMC133

Fig. 3

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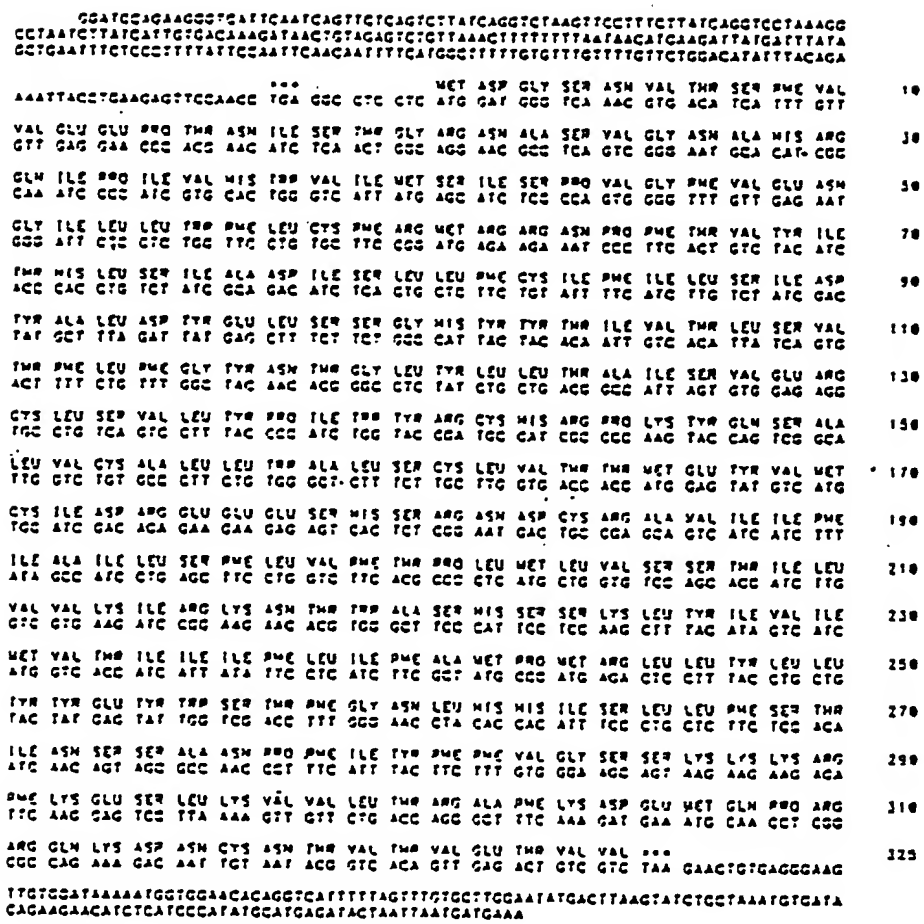

Fig. 4

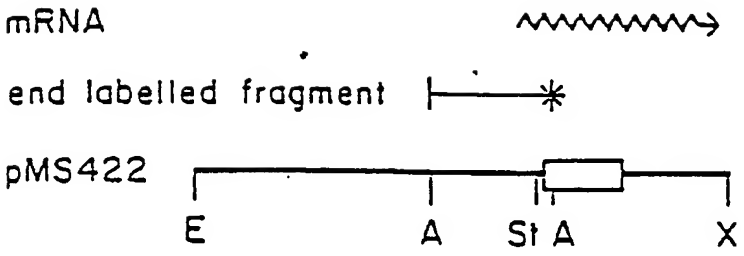

Fig. 5

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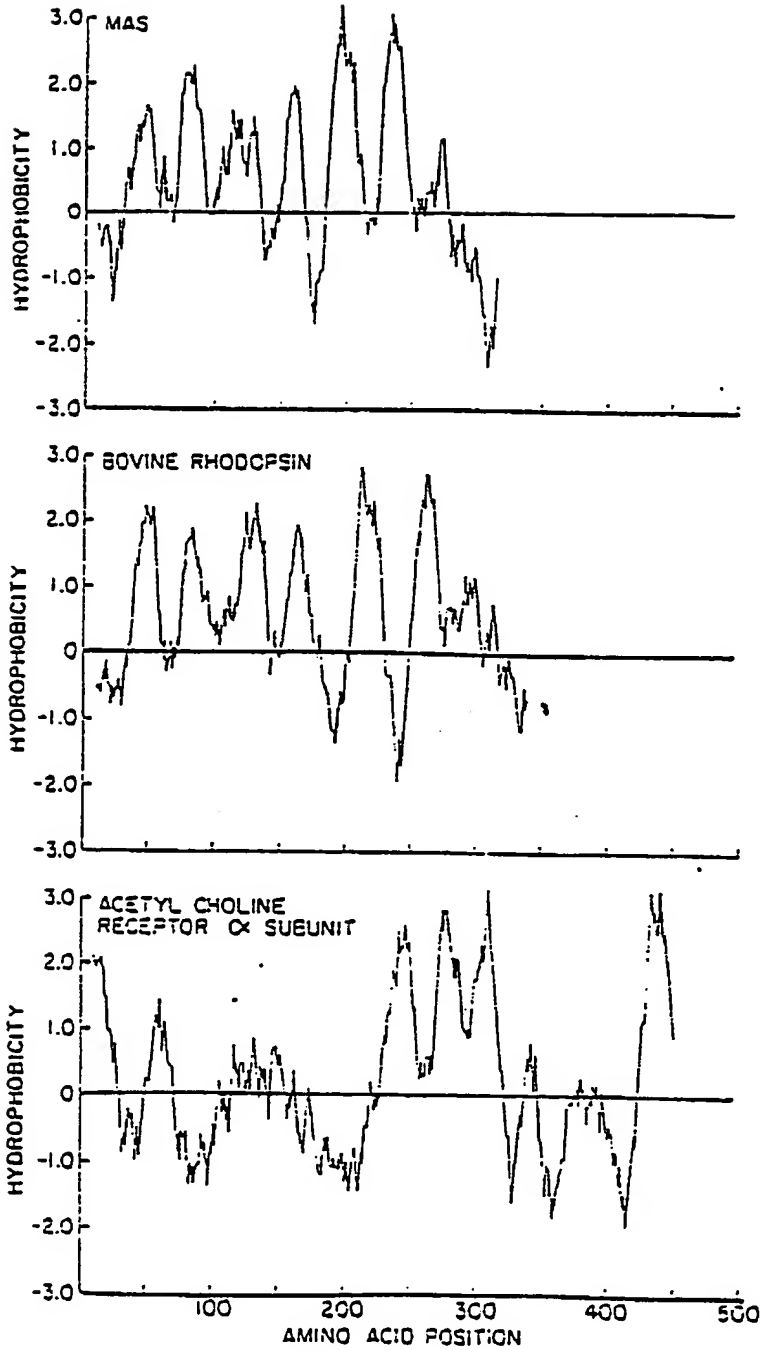
MAS
BOVINE RHODOPSIN
ACETYL CHOLINE
RECEPTOR α SUBUNIT
HYDROPHOBICITY
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400
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AMINO ACID POSITION

PCT

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(88) Date of publication of the international search report:
30 December 1987 (30.12.87)

(54) Title: A DNA SEQUENCE ENCODING THE *MAS* ONCOGENE, POLYPEPTIDES ENCODED THEREFROM, AND DIAGNOSTIC AND OTHER METHODS BASED THEREON

(57) Abstract

A DNA sequence comprising an activated oncogene, said oncogene encoding a polypeptide capable of transforming NIH3T3 cells and of inducing a tumor when injected into nude mice, said DNA sequence having a nucleotide sequence substantially as shown in Fig. 3. The invention also concerns a polypeptide molecule encoded by an activated oncogene, said molecule having the properties of transforming NIH3T3 cells and of inducing a tumor when injected into nude mice and further said polypeptide having an amino acid sequence substantially as shown in Fig. 3. Finally, this invention provides a method for treating a tumor induced by an activated *mas* oncogene.

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INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/01347

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl(4): C12N 15/00;C12Q 1/68;G01N 33/53;C08G 73/00;C07K 15/00
U.S.Cl: 424/7.1,85;435/6,7,172.2; 530/403,413; See Continuation.

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	424/7.1,85;435/6,7,172.2;530/403,413;536/27 436/64,501,504,519,542;935/2,3,6,9,12,78,81

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

Computer Searches: Chemical Abstracts Online, BIOSIS, MEDLINE, Automated Patent System (1975-present)

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US, A, 4,460,559 (GOLDENBERG) 17 July 1984, see column 2, lines 18-31, 46-51; column 4, lines 4-8; column 6, lines 40-44; column 13, line 53- column 15- line 11.	52,64
Y/A	US, A, 4,535,058 (WEINBERG ET AL) 13 August 1985, see column 3, lines 38-45; column 5, line 54- column 6, line 21; column 10, lines 42-50; column 16, line 54- column 17, line 59.	4-16,20-33, 39-41,46-49 / 17-19,34-35, 42-45,50-51, 53-62
Y/A	US, A, 4,542,096 (LEDER) 17 September 1985, see column 1, lines 5-12; column 2, line 61- column 3, line 4.	4-6,8-9, 20-22, 24-25 / 7,10-19, 23,26-35

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
10 NOVEMBER 1987	01 DEC 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	Richard W. Wagner Richard W. Wagner

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	EP, A, 0,108,564 (The Reagents of the University of California) 16 May 1984, see all claims.	1-64
Y A	WO 84/03087 (SEN ET AL) 16 August 1984, see page 6, lines 29-33; page 7, lines 1-32; page 8, lines 16-35; page 43, lines 9-29.	36,39-48, 51-54, 56-58, 60-64 37-38, 49-50, 55,59
Y A	Proceedings of the National Academy of Sciences, Vol. 80, issued April 1983 (The National Academy of Sciences, Washington, DC 20418), K. Shimizu et al, "Three human transforming genes...", pages 2112-2116, see abstract.	4-9,20-25, 39-48, 53-57 10-19,26-35, 49-51, 58-62
Y A	Molecular and Cellular Biology, Vol. 4, No. 9, issued September 1984 (American Society for Microbiology, Washington, DC 20006), O. Fasano et al, "New Human Transforming Genes...", pages 1695-1705, see page 1696, column 1, lines 5-20.	4-9,20-25 10-19, 26-35
Y	Journal of Immunological Methods, Vol. 85, issued September 1985 (Elsevier Science Publishers, Amsterdam, The Netherlands), R.H. Kennett et al, "Detection of E. coli Colonies...", pages 169-182, see Abstract, page 180, lines 15-30.	39-51, 53-62

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y A	US, A, 4,559,311 (STENMAN ET AL) 17 December 1985, see column 1, line 46-column 2, line 2; column 4, lines 48-51.	36,39-51, 53-62 37-38
Y,P A,P	US, A, 4,599,305 (WITTE ET AL) 08 July 1986, see column 3, lines 28-50; column 4, lines 59-68; column 8, lines 28-56.	36,39-51, 53-55, 58-60,63 37-38, 56-57,62

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [10]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers ... . . because they relate to subject matter [12] not required to be searched by this Authority, namely:

2. ☐ Claim numbers....., because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [13], specifically:

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [11]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

PCT/US87/01347

I. CLASSIFICATION OF SUBJECT MATTER (CONTINUED)

U.S.Cl.: 536/27;436/64,501,504,519,542;
935/2,3,6,9,12,78,81